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Development of a Molecular-Beacon Assay To Detect the $G_{1896}A$ Precore Mutation in Hepatitis B Virus-Infected Individuals

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The 1896 precore (PC) mutation is the most frequent cause of hepatitis B virus e-antigen (HBeAg)-negative chronic hepatitis B virus (HBV) infection. Detection of the 1896 PC mutation has application in studies monitoring antiviral therapy and the natural history of the disease. Identification of this mutation is usually performed by direct sequencing, which is both costly and laborious. The aim of this study was to develop a rapid, high-throughput assay to detect the 1896 PC mutation using real-time PCR and molecular-beacon technology. The assay was initially standardized on oligonucleotide targets and plasmids containing the wild-type (WT) and PC mutation and then tested on plasma samples from children with HBV DNA of $>10^6$ copies/ml. Nine individuals were HBeAg negative and suspected to harbor HBeAg mutations, while 12 children were HBeAg positive and selected as controls. Ninety percent (19 of 21) of plasma samples tested with molecular beacons were in complete agreement with sequencing results. The remaining 10% (2 of 21) of samples were identified as heterogeneous mixtures of WT and mutant virus by molecular beacons, though sequencing found only a homogeneous mutant in both cases. Overall, the 1896 PC mutation was detected by this assay in 55.5% of the children with HBeAg-negative infection. In summary, this assay is a rapid, sensitive, and specific technique that effectively discriminates WT from 1896 PC mutant HBV and may be useful in clinical and epidemiological studies.

Approximately 350 million individuals are chronically infected with the hepatitis B virus (HBV) worldwide (14, 20, 21, 23). HBV is the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) and is responsible for 60 to 80% of all HCC cases and more than 1 million deaths per year (20, 33, 34). The clinical course of HBV infection is monitored by biological markers in the serum, including liver enzymes such as alanine aminotransferase, and direct quantification of HBV DNA viral load. Seroconversion to HBV e-antigen (HBeAg)-negative and e-antibody (HBeAb)-positive status usually implies a decrease in viral replication, decrease in potential liver injury, improved prognosis, and decrease in HCC risk (16) and is the major end point of therapy (10, 29). However, in many cases the patient remains viremic, exhibiting active liver disease without presence of HBeAg. In most instances this is a result of HBV variants that produce little or no HBeAg.

The HBeAg is encoded by the precore (PC) and core genes. The PC open reading frame codes for a hydrophobic leader peptide that directs the PC-core protein to the endoplasmic reticulum for posttranslational modification (25). Further processing of the protein at the C terminus generates HBeAg, which is secreted into the serum (10, 29). Variations in the PC region affect HBeAg synthesis without appreciably affecting HBV replication. These variants often contain mutations within the PC or core promoter region that decrease or prevent HBeAg production. However, the most common cause (60 to 80%) of HBeAg-negative variants is the 1896 guanine (G)-to-adenine (A) PC mutation, which produces a stop codon in the PC open reading frame and prevents HBeAg synthesis (4, 14). In addition to the 1896 PC mutation, basal core promoter variants, such as A1762T and G1764A, may also downregulate HBeAg production (1, 18, 19).

The standard method to characterize PC variants is PCR and direct sequencing. However, sequencing has limitations in detecting low quantities (<20%) of mutant virus within heterogeneous mixtures and is tedious, time-consuming, and expensive. Although a line-probe assay was recently developed to detect the 1896 PC mutation (6, 18), currently there are no Food and Drug Administration-approved procedures for this purpose.

The aim of this study was to develop a rapid, sensitive, and inexpensive real-time PCR and molecular beacon assay for detection of the 1896 PC mutation. Molecular-beacon technology was utilized because it is a novel, robust technique capable of detection of single nucleotide polymorphisms (SNPs) such as the 1896 PC mutation (24). The assay was tested in a population of HBeAg-negative and HBeAg-positive pediatric patients with elevated HBV DNA. The assay sensitivity in homogeneous and mixed populations of HBV was assessed, and the prevalences of the PC and basal core promoter mutations in this population are reported.

MATERIALS AND METHODS

Molecular beacons, primers, and oligonucleotide targets. Two molecular beacons were constructed to hybridize to the region surrounding the 1896 PC sequence (Table 1). These molecular beacons hybridized to their target within a 104-bp fragment, amplified by the PB primers described in Table 1 (9). The two molecular beacons utilized contained the wild-type (WT) G1896 and mutant A1896 sequences. They were produced according to the methods described by

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TABLE 1. Sequences of molecular beacons, primers, and oligonucleotides

Beacon, primer or oligonucleotide	Sequence (5′–3′)	Nucleotides
Molecular beacon		
WT	TET-5′-CGTCCG *CTTTGGGGCATGGACATTG*ACGGACG-3′-DABCYL	1892–1911
Mutant	FAM-5′-CGTCCG *CTTTAGGGCATGGACATTG*ACGGACG-3′-DABCYL	1892–1911
Primers		
PB1fw	GTTCAAGCCTCCAAGCTGTG	1862–1881
PB2 rv	TCAGAAGGCAAAAAAGAGAGTAACT	1965–1941
PR2 fw	TACTTCAAAGACTGTGTGTTTA	1704–1725
PR2 rv	CTCCACAGTAGCTCCAAATTC	1942–1922
Oligonucleotides		
WT PC	TCAATGTCCATGCCC**C**AAAGCC	1911–1890
Mut PC	TCAATGTCCATGCCC**T**AAAGCC	1911–1890

Tyagi and Kramer (24, 32). The intramolecular configuration of the molecular beacons was modeled using the Zuker program (35, 36). Both molecular beacons included a 6-bp stem sequence, with the DABCYL quencher linked to the 3′ end and the 6-carboxytetrachlorofluorescein (TET) or 6-carboxyfluorescein (FAM) fluorophore linked to the 5′ end of the WT and mutant molecular beacons, respectively. Oligonucleotide targets were created to test the functionality of the molecular beacons (Table 1).

Real-time PCR conditions. DNA was extracted from 200 μl of plasma with a QIAamp DNA mini kit (QIAGEN), eluted in 50 μl of elution buffer, and stored at −70°C. The amplification-detection was carried out in an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, Calif.). The PB primers amplified a 104-bp amplicon in the PC region. The reaction was carried out in a final volume of 25 μl consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, a 200 μM concentration each of dATP, dGTP, dTTP, and dCTP, a 0.5 μM concentration of each of the primers, 0.25 μmol of TET WT molecular beacon, 0.25 μmol of FAM mutant molecular beacon, 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems), and 5 ng of DNA. The amplification procedure consisted of 95°C for 10 min followed by 40 cycles of 95°C for 20 s, 62°C for 30 s, and 72°C for 30 s. The fluorescence spectra of the molecular beacons were measured during the annealing step of the PCR cycle. The ABI Prism 7700 sequence detection system software (SDS version 1.7) plotted TET and FAM intensity values versus cycle number. The PR primers were used to amplify a 239-bp fragment in the core promoter and PC regions for subsequent sequencing, using the same amplification conditions without molecular beacons (Table 1) (12).

Thermal denaturation profiles. To demonstrate that the molecular beacons readily distinguished targets that differed by a single base pair at nucleotide 1896 and to determine the optimal annealing temperature, a thermal denaturation profile was created (2, 24, 26). The amount of fluorescence of each molecular beacon, in the presence or absence of synthetic target oligonucleotides, was measured as a function of temperature. Solutions of 50 μl contained 50 nM molecular beacon, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and either no target, 300 nM complementary oligonucleotide target, or 300 nM mismatched oligonucleotide target (Table 1). The reaction was performed in the ABI Prism 7700 sequence detection system. Reaction conditions included a 15-s, 1°C stepwise increase in annealing temperature from 25 to 95°C.

Plasmid constructs. Plasmid constructs *payw* and *payw*28* previously described by Scaglioni et al. (29) and which contain the WT and PC mutation sequences at nucleotide 1896, respectively, were utilized to determine the sensitivity and specificity of the assay. Once these parameters were assessed, the plasmids were used as positive and negative controls during subsequent analyses of clinical specimens.

Patient samples. The 21 plasma samples used for this study were selected from a pediatric population (<18 years of age) of 269 HBV-positive patients who were followed by the Division of Pediatric Infectious Diseases at Bellevue Hospital and the NYU School of Medicine from 1995 through 2003. The mean age at first evaluation of this population was 14.37 ± 3.49 years (mean ± standard deviation) for HBeAg-negative patients and 13.14 ± 4.43 years for HBeAg-positive patients. Individuals were selected if plasma specimens were available and if serology indicated a high risk of the 1896 PC mutation. The criteria that defined a high risk of the 1896 PC mutation were HBeAg negative, HBeAb positive, and HBV DNA viral load levels of $>10^6$ copies per ml of serum at the time the sample was processed. Nine individuals met these criteria and were suspected to harbor the 1896 PC mutant virus. Twelve patients with presumed WT virus were also selected as controls based on the following criteria: HBeAg positive, HBeAb negative, and HBV DNA viral load levels of $>10^6$ copies per ml of serum.

All plasma samples tested were excess, archived, frozen samples from individuals enrolled in other NYU School of Medicine Institutional Review Board-approved protocols. After an aliquot of plasma was placed into a new tube, all personal identifiers were removed and replaced with a study number that was linked to deidentified clinical data consisting of HBV viral load and HBeAg-HBeAb status. HBV DNA isolated from these samples was then sequenced, and the molecular-beacon assay was performed. The storage and collection of study data and patient samples conformed to all HIPAA regulations. The protocol was approved by the respective Institutional Review Boards for the NYU School of Medicine and Bellevue Hospital.

Sequencing. Amplified product (nucleotides 1704 to 1942) was sequenced at the DNA Sequencing Facility located in the Skirball Institute of Biomolecular Medicine. The Big-Dye Terminator version 1.1 Ready Mix and ABI 377XL sequencer were utilized. PC, basal core promoter, and other mutations were mapped for each individual.

Data analysis. Specificity was assessed with homogeneous samples of WT *payw* and 1896 PC mutant *payw*28* plasmids. To assess the sensitivity of the assay, the lower limit of detection for each molecular beacon was found using serial 10-fold end point dilutions of the target plasmid. The sensitivity of the assay in heterogeneous samples of WT and 1896 PC mutant was determined for each molecular beacon with mixed virus populations and compared with sequencing results. Initial mixed dilution experiments were performed in duplicate and in increments of 5%. Subsequently, a finer-dilution experiment was performed in 1 to 2% increments. The prevalence of the 1896 PC mutation detected by the molecular-beacon assay was compared to that detected by sequencing. The prevalence of basal core promoter mutations identified by sequencing was also reported.

RESULTS

Molecular-beacon performance parameters. (i) Thermal denaturation profiles. Optimal hybridization of molecular beacons to the target sequence occurred at an annealing temperature of 62°C. At this temperature, maximum discrimination for the complementary oligonucleotide target occurred, and high fluorescence was measured with little or no background for mismatched targets. The thermal denaturation profile for the WT molecular beacon is displayed in Fig. 1.

(ii) Results and standardization with plasmid constructs. The molecular beacons accurately detected their corresponding plasmids. Specificity of the assay within homogeneous populations when tested using the *payw* and *payw*28* plasmids was 100% for each of the molecular beacons.

Tenfold-dilution experiments demonstrated that the lower

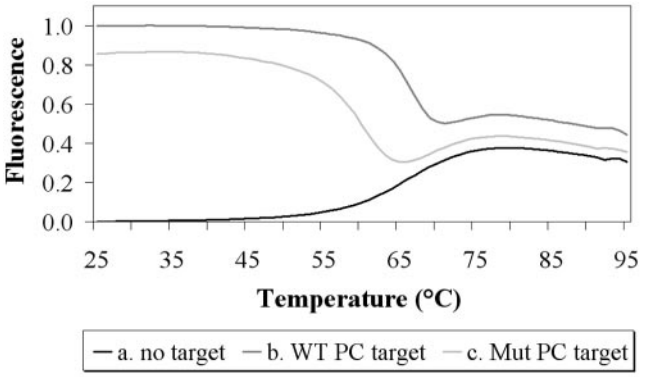

FIG. 1. Thermal denaturation profile of solutions containing WT molecular beacon in the absence of targets (a), in the presence of perfectly complementary oligonucleotide (WT PC) (b), and in the presence of single-nucleotide-mismatched oligonucleotide (Mut PC) (c).

limit of detection of the WT molecular beacon was 10^2 copies/ml, and the mutant molecular-beacon detection limit was 10^1 copies/ml. The inverse linear relationship between the threshold cycle (*Ct*) and the logarithm of starting quantity of DNA demonstrated that the threshold cycle for each 10-fold dilution was approximately three cycles greater than that of the previous dilution (Fig. 2).

Mixed dilution experiments of WT and mutant plasma DNA revealed that the WT molecular beacon detected as low as 2% of the WT DNA when combined with 98% mutant. The mutant molecular beacon detected as low as 15% mutant DNA when mixed with 85% WT DNA (data not shown). Once the molecular beacons' functionalities and performance parameters were determined, the assay was used to examine patient DNA samples.

Performance on clinical samples. All 21 DNA samples were tested using the molecular-beacon assay. An example of the assay performance is illustrated in Fig. 3. The results of the molecular-beacon assay are presented in Table 2, along with sequence results of the patient samples. The molecular-beacon results correlated with direct sequencing results for samples 1 to 12, 14 and 15, and 17 to 21. Samples 13 and 16 were found to contain mixtures of WT and mutant virus by molecular

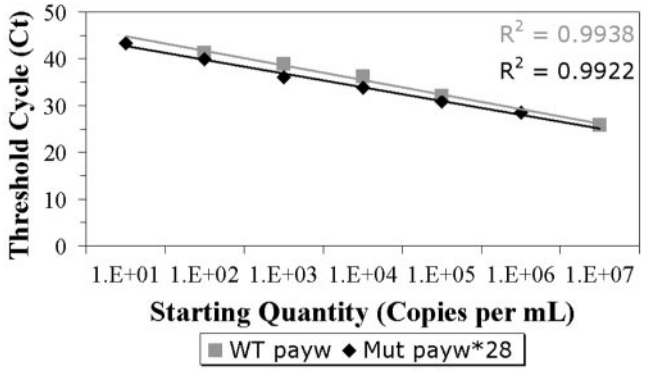

FIG. 2. Sensitivity of the molecular-beacon assay as determined by a series of dilutions of WT *payw* plasmid and a series of dilutions of mutant *payw*28* plasmid.

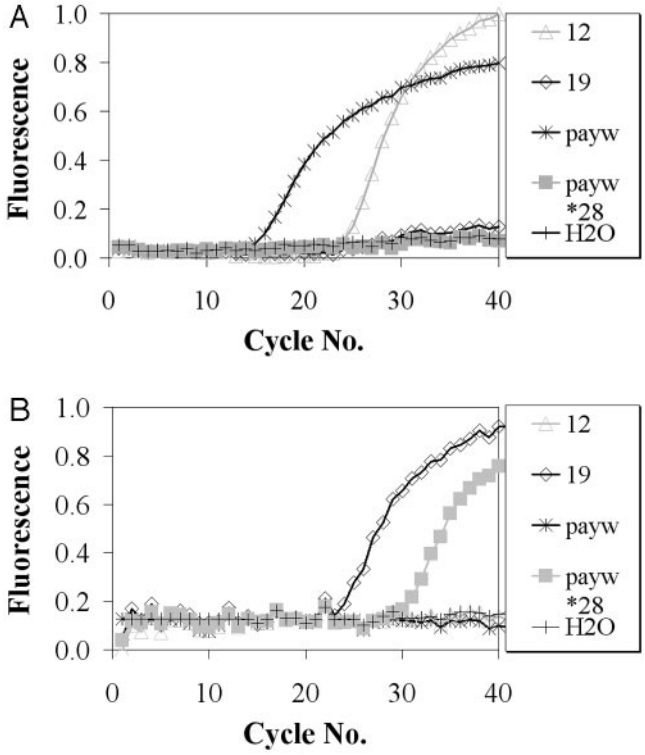

FIG. 3. Real-time PCR results for 2 of the 21 DNA samples and WT and mutant plasmids. (A) With the WT (TET) molecular beacon, fluorescence exceeded threshold values for DNA sample 12 and the WT *payw* plasmid (10^7 copies/ml). (B) The mutant (FAM) molecular beacon detected fluorescence above threshold values for DNA sample 19 and the mutant *payw*28* plasmid (10^5 copies/ml).

beacon, though direct sequencing only found adenine (mutant) at nucleotide 1896.

The prevalence of 1896 PC mutant and WT virus in the clinical samples as measured by the molecular-beacon assay was determined. WT virus was found exclusively in 12 of 12 samples from HBeAg-positive individuals. In contrast, among the HBeAg-negative samples, the PC mutation was found exclusively in two of nine samples, while three of nine specimens contained mixtures of WT and mutant virus. Overall, 55.5% of HBeAg-negative patient samples had evidence of the PC mutation, while 44.4% (four of nine) contained only pure WT virus. The prevalence of basal core promoter mutations identified by sequencing was 1 of 12 (8%) in HBeAg-positive patients and 5 of 9 (55.5%) in HBeAg-negative patients (Table 2). One of the nine (11%) HBeAg-negative individuals harbored virus that contained both the 1896 PC and 1762/1764 basal core promoter mutations.

DISCUSSION

The results of this study indicate that the molecular beacons developed for this assay can accurately distinguish between 1896 PC WT and mutation sequences. High sensitivity and specificity were demonstrated by 10-fold end point dilutions and mixed dilutions, with a lower limit of detection of 10 to 100 copies of virus per ml of serum and 2 to 15% within a mixed virus population. The observed data additionally indicate that

TABLE 2. Results of molecular-beacon assay and sequencing for each sample

Sample no.	e-A status[a]	HBV DNA (copies/ml)	Real-time PCR result		Sequencing result		
			WT[b]	Mut[b]	G1896A[c]	A1762T[c]	G1764A[c]
1	+	4.30E+09	+	−	G	T	A
2	+	4.80E+09	+	−	G	A	G
3	+	3.10E+09	+	−	G	A	G
4	+	6.30E+09	+	−	G	A	G
5	+	3.80E+09	+	−	G	A	G
6	+	5.30E+09	+	−	G	A	G
7	+	4.10E+09	+	−	G	A	G
8	+	1.20E+09	+	−	G	A	G
9	+	4.30E+09	+	−	G	A	G
10	+	1.10E+09	+	−	G	A	G
11	+	5.40E+08	+	−	G	A	G
12	+	1.40E+09	+	−	G	A	G
13	−	7.00E+06	+	+	A	T	A
14	−	4.40E+07	+	−	G	T	A
15	−	1.90E+08	+	−	G	T	A
16	−	1.60E+07	+	+	A	A	G
17	−	1.60E+06	−	+	A	A	G
18	−	6.00E+07	+	−	G	T	A
19	−	5.70E+08	−	+	A	A	G
20	−	1.20E+08	+	−	G	T	A
21	−	3.90E+08	+	+	G and A	A	G
payw			+	−	G		
*payw*28*			−	+	A		

[a] +, HBeAg positive; −, HBeAg negative.
[b] +, fluorescence exceeded threshold values for molecular beacons; −, fluorescence did not exceed threshold values for molecular beacons.
[c] G, guanine; A, adenine; T, thymine.

this molecular-beacon assay is more sensitive than direct sequencing, as mixtures of WT and mutant virus were identified in samples 13 and 16, for which sequencing only found mutant virus. These findings are consistent with other reports that denote the high specificity and sensitivity of molecular beacons used to detect SNPs (2, 24, 26, 32). By contrast, direct sequencing is limited in its ability to detect small quantities of a strain within a mixed population, thus leading to false-negative results (8, 30).

The overall prevalence of the 1896 PC mutation in the pediatric patients in this study with HBeAg-negative status was 55.5%. In 22.2% only the 1896 PC mutation was found, whereas in 33.3% the mutation was present along with WT virus. Homogeneous WT virus was found in all of the HBeAg-positive patients. These results fall into the range of prevalence indicated by other epidemiology studies in adults. One epidemiology study found the median prevalence of the 1896 PC mutation in HBeAg-negative adults to be 50% in Asia, 92% in the Mediterranean, and 24% in America (11). The frequency of the 1896 PC mutation varies geographically and primarily depends on the genotype of HBV (14, 17). The 1896 PC mutation is most prevalent in non-A or non-F genotypes, due to a genotype-specific nucleotide difference upstream from 1896 that can critically alter the secondary conformation of the HBV encapsidation signal (22, 25, 28). Although PC variants predominate in the Mediterranean (non-A genotypes), there is an increasing prevalence worldwide, particularly of genotypes B and C in Asia (14, 17).

The 1896 PC mutation, although the most common, is not the only cause of HBeAg-negative virus. Other mutations that result in an HBeAg-negative phenotype or diminish HBeAg production include mutations in the basal core promoter (A1762T and G1764A) (19) and in the PC start codon (1) or deletions-insertions in the PC region (13). Sequencing of the 21 patient samples in this study revealed basal core promoter mutations in 55.5 and 8% of HBeAg-negative and HBeAg-positive individuals, respectively. All HBeAg-negative individuals in this study were found to contain virologic mutations that are associated with decreased HBeAg production. The prevalence of basal core promoter variants in HBeAg-negative pediatric patients in this population is in accordance with previous reports; however, the frequency of these variants in HBeAg-positive patients in this study varies from reports in adults (6, 18). This apparent difference between adult and pediatric patients merits further investigation.

Comprehensive studies of the PC mutation have particular application in monitoring antiviral therapy and the natural history of the disease. PC mutants are reported to increase replication efficiency in lamivudine-resistant HBV in vitro. HBeAg-negative, lamivudine-resistant HBV has been shown to produce higher HBV DNA levels and may be more cytopathic (5). Longitudinal studies of patients with a mixed virus population have shown a dynamic fluctuation of WT and mutant virus, driven by immune pressure (3, 15, 18, 25). The temporal emergence of HBeAg-negative variants relative to seroconversion has been found to critically affect the course of chronic HBV infection (7). An assay that can detect low quantities of virus within a heterogeneous viral population would therefore be useful in studies regarding viral kinetics and emergence of the PC mutants longitudinally.

The rapidity and high capacity of this assay enable detection of PC mutants for up to 96 samples within 3 h (27). The molecular-beacon assay is inexpensive, rapid, specific, and simple compared to other assays used to detect the 1896 PC mutation, such as direct sequencing, primer-extension assays, allele-specific PCR, restriction fragment length polymorphism analysis, and line-probe assays (2, 18, 31). This assay is also less susceptible to contamination, because amplification, hybridization, and analysis are all performed in sealed wells (24, 27, 32). Molecular beacons, however, do have their limitations, which must be considered. Since they can distinguish SNPs, other mutations or polymorphisms in the region of the probe may prevent hybridization and therefore lead to indeterminate results. Furthermore, although the PC region is generally conserved among genotypes, this assay needs to be validated for genotypes E, F, and G. Under these circumstances, sequencing may prove beneficial by fully characterizing PC, basal core promoter, and other mutations, which may also have implications in the natural history of chronic HBV infection.

In summary, the molecular-beacon assay described in this paper is a rapid, inexpensive, and sensitive method of detecting the 1896 PC mutation. The high sensitivity of this assay (10 to 100 copies/ml) indicates its utility even in patients with low HBV DNA levels. The high sensitivity and ability to detect WT or PC status in heterogeneous populations of virus will have particular value in longitudinal studies. Although many attributes of PC mutants have been described, the clinical significance of these mutants in chronic HBV infection has not been entirely elucidated. There remains a need for a systematic evaluation of the role of PC mutants in chronic HBV infection.

This assay, in conjunction with sequencing, may assist in the need to define the clinical relevance of PC mutants and other HBeAg-diminishing mutations, as well as further define the impact of infecting HBV genotype. This methodology may also be remarkably beneficial in studies of the epidemiology and natural history of PC mutants in HBV-infected patients.

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